academicJournals

Vol. 6(12), pp. 181-188, December 2014
DOI: 10.5897/JPVB2014.0177
Article No: 7F6B73549599
ISSN 2141-2510

http://www.academicjournals.org/JPVB

Journal of Parasitology and Vector Biology

Full Length Research Paper

Development and evaluation of lateral flow test for the detection of trypanosomes in tsetse flies

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Received 16 October, 2014; Accepted 22 December, 2014

Lateral flow test (LFT) which is based on antibodies raised against the *Trypanosoma brucei* peptide was evaluated for its analytical sensitivity, specificity, positive predictive value and negative predictive values in order to assess its utility for surveillance of trypanosome infections in tsetse flies. The diagnostic test agreement of the FLT and microscopy was 91.0% and kappa value of 80.5% at the confidence interval 0.72 to 0.80 which is a high level of test agreement. Furthermore, the relative diagnostic sensitivity and specificity of lateral flow test were 83.0 and 96.0%, respectively. Both the positive predictive and the negative predictive values were high at 92.7 and 90.0%, respectively. The LFT is, therefore, recommended for surveillance of trypanosomes in tsetse flies in order to: (i) indicate areas with tsetse infected with trypanosomes; (ii) indicate tsetse infected with potentially human pathogenic trypanosomes and (iii) guide in prioritizing control strategies for human African trypanosomiasis (HAT).

Key words: Lateral flow test, detection, xenomonitoring, trypanosomes, tsetse flies.

INTRODUCTION

Tsetse fly-transmitted african trypanosomes (*Trypanosoma brucei*) are major pathogens of humans and livestock, causing Human African Trypanosomiasis (HAT) and Nagana, respectively. In Eastern and Southern Africa, including Western and Central Africa, trypanosomes are transmitted by various species of tsetse flies, the most important being *Glossina fuscipes fuscipes*, *Glossina pallidipes* and *Glossina morsitans*

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morsitans. Animal trypanosomiasis (Nagana) is one of the major constraints in livestock production in Sub-Saharan Africa. Current losses due to trypanosomosis in cattle are valued at 4.5 billion dollars (U.S.) per year, with about 3 million cattle deaths (Budd, 1999), while farmers spend about US $35 million annually on nagana treatment.

Human trypanosomiasis (HAT) also called sleeping sickness affects people in 37 of 52 countries in Africa. The most highly endemic/epidemic countries are: Democratic Republic of Congo, Congo Republic, Angola, Central African Republic, Southern Sudan, Uganda and Tanzania. About 60 million people in Sub-Saharan Africa are at risk of sleeping sickness of whom about 4 million people are under surveillance and about 300,000 sleeping sickness cases are reported yearly. It is estimated that about 40,000 people diagnosed and treated and the rest left undiagnosed/untreated and a case fatality rate in untreated cases is 100% (WHO Fact sheet No 259, 2014). *T. brucei gambiense* is found in West and Central Africa. This form currently accounts for over 98% of reported cases of sleeping sickness and causes a chronic infection. A person can be infected for months or even years without major signs or symptoms of the disease. The second type of HAT called *T. brucei rhodesiense* is found in Eastern and Southern Africa. Nowadays, this form represents under 2% of reported cases and causes an acute infection. First signs and symptoms are observed a few weeks or months after infection. The disease develops rapidly and invades the central nervous system. Only Uganda presents both forms of the disease (WHO, Fact sheet No 259 of March, 2014). However, other parasite species and sub-species of the *Trypanosoma* genus are pathogenic to animals and cause animal trypanosomiasis in wild and domestic animals. In cattle the disease is called *Nagana*.

The pathway taken by trypanosomes through the tsetse fly is complicated and the flies have low infection rates even in endemic areas (Okoth and Kapata, 1986). Furthermore, it is difficult to differentiate the types of trypanosomes pathogenic to humans from those non-infective ones that circulate in tsetse flies. Trypanosomes of different species undergo cycles of development of varying complexity within the tsetse fly, transforming from bloodstream forms to procyclic non-mammalian infective forms in the fly midgut. *T. brucei* undergoes complex cycles of differentiation and multiplication in the tsetse fly. Flies are refractory to infection and the bloodstream form trypanosomes in the infected feed differentiate into viable procyclics. Different life cycle stages of *T. brucei* are: (a) Procyclic form in fly midgut, (b) long trypomastigote in fly proventriculus, (c) asymmetric dividing epimastigote in fly proventriculus, (d) short epimastigote in fly proventriculus, (e) attached epimastigotes in fly salivary gland, (f) metacyclic trypanosome in salivary gland, prepared for host inoculation. The 3 subspecies *T. brucei brucei*, together with the causative organisms of human African trypanosomiasis, *T. b. rhodesiense and T. b. gambiense*, must migrate from the midgut of the tsetse fly and transform to infective metacyclic forms in the salivary glands. Only a proportion of midgut infections mature into salivary gland infections with male tsetse maturing significantly more midgut infections than females (Dale et al., 1995; Milligan et at., 1995). Trypanosome genotype also influences maturation; those resistant to human serum (*T. b. rhodesiense*) are less likely to produce mature infections than human serum sensitive (*T. b. brucei*) parasites (Welburn et al., 1995). The mature *T. brucei* called the metacyclic form in the tsetse salivary glands are the ones regurgitated during the bite of the fly to the mammalian hosts.

A crucial component in HAT vector control and monitoring programmes is the sensitive detection of human-infective trypanosome species in the tsetse fly vector. The infection status of a tsetse fly is usually assessed by the presence/absence of trypanosomes by dissection and microscopic examination to identify the trypanosomes in the mouthparts, mid-guts and salivary glands. This is tedious and laborious.

It has already been demonstrated that it is possible to extract and amplify trypanosome DNA from whole tsetse flies (Ferreira et al., 2008). Detection of trypanosomes that cause disease in human beings and livestock within their tsetse fly hosts is an essential component of vector and disease control programmes. Several molecular-based diagnostic tests have been developed for this purpose (Malele et al., 2013). Many of these tests, while sensitive, require analysis of trypanosome DNA extracted from single flies. Previous serological tests used in diagnosis of HAT are immune-fluorescent test (IFAT) by Wery et al. (1970); enzyme linked immunoabsorbent (ELISA) by Komba et al. (1992) and Nantulya et al. (1987); card agglutination test (CATT) by Magnus et al. (1978); card indirect agglutination test for trypanosomiasis (CIATT) by Asonganyi et al. (1998) which are cumbersome to perform and have various sensitivities and specificities. However, recently developed LFT is an antigen test which is simple to perform and detects circulating *T. brucei* antigens in tsetse flies. In LFT, a filter paper is coated with antibodies against *T. brucei* and when homogenized tsetse midgut sample is added to the filter, agglutination is produced if trypanosome antigens of *T. brucei* are present.

The aim of this study was to evaluate LFT for detection and xenomonitoring of trypanosomes in tsetse flies under field conditions.

MATERIALS AND METHODS

Tsetse fly collection for trypanosome screening

Cross sectional surveys were done between April to July, 2013 in selected sleeping sickness endemic areas in each of the 6 countries (Uganda in Alwa, Dokolo, Tororo and Omugu, Tanzania in Serengeti, and Urambo, Malawi in Nkhotakota and Liwonde, Democratic Republic of Congo in Kwamouth and Itubi, South

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Clone2       MFVFICRDFVSFFPLLSLFIPFFLFLLFHFECPYVSKCFLLIEDCPWQFLSYHFIFLSLV 60
TB927-Target MFVFICRDFVSFFPFLSLFIPFFLFLLFHFECPYVSKCFLLIEDCPWQFLSYHFIFLSLV 60
Clone4       MFVFICRDFVSFFPLLSLFIPFFLFLLFHFECPYVSKCFLLIEDCPWQFLSYHFIFLSLV 60
Clone3       MFVFICRDFVSFFPLLSLFIPFFLFLLFHFECPYVSKCFLLIEDCPWQFLSYHFIFLSLV 60
             ************.************************************************

Clone2       RFILYHFSFRFILYHFSFRFILYHFSFRFIYYFILLLFLFLFR---------CSHTH--- 108
TB927-Target LFVLYHFSFRFILYHFSFRFILYHFSFRFIYYFILLSFHLLFYFTFVSIFVSAAHTHT-H 119
Clone4       RFILYHFSFRFILYHFSFR---------FIYYFILLLFLFLFP--------LLTYTHTPC 103
Clone3       RFILYHFSFRFILYHFSFRFILYHFSFRFIYYFILLLFLFLFP--------LLTYTHTPC 112
             *.****************     ******** * .**      ..**

Clone2       ----------
TB927-Target THTLLFQLHDIITH 133
Clone4       SSSCMTSLHT---- 113
Clone3       SSSCMTSLHT---- 122
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Figure 1. CLUSTAL 2.0.12 multiple sequence alignment of Tb927.1.4620 and orthologues Clones 2, 3, and 4.

Sudan and Sudan in Kajokeji, Yambio and Tambura). The rest of the work was done in the laboratories in each country.

Fly dissection to screen trypanosomes

All live flies were dissected as described in Msangi et al. (1998). Midguts found positive by microscopy were transferred individually into labelled vials for analysis by TBR-PCR, ITS-PCR, LAMP and lateral flow tests.

Development of lateral flow test

Trypanozoon markers were selected using the *T. brucei* genome (*Trypanosoma brucei* TREU927), the *T. gambiense* (Tbg 972) genome, *T. congo*, and *T .vivax* genomes all contained in Tritryp data base (tritrypdb.org/). The criteria used was: (a) mining the genes only peculiar to the *Trypanozoon* parasites and (b) further checking *in silico* for mRNA expression profiles as an indicator that the gene phenotype is present. One of these genes which was selected was Tb927.1.4620 and its orthologues and specific primers were designed to confirm its unique presence in *Trypanozoon.*

Uninfected tsetse midguts from teneral and non-teneral flies for assessing specificity of lateral flow test

Midguts from 45 teneral and 25 non-teneral tsetse were tested to assess specificity of the lateral flow test. The samples were tested singly by both microscopy and lateral flow tests.

Experimentally infected tsetse flies for assessing sensitivity of lateral flow test

Midguts from a total of 128 experimentally infected tsetse flies were analyzed by both microscopy and Lateral Flow tests.

Statistical analysis

Statistical analyses were performed using Prism version 5.0 GraphPad Software (GPW5, USA) and StatMate version 2.0 GraphPad Software (GSW5, USA). The sensitivity and specificity of lateral flow assays were calculated from data entered into contingency tables. Sensitivity was defined as the proportion of positive cases that are positive by the LFT test and specificity as the proportion of controls that are negative by the LFT. Differences in sensitivity and specificity between the different tests were estimated by the Mc Nemar test while differences between LFT and microscopy were estimated with the Fisher exact test, setting the level of significance at 0.05. Agreement between the two tests was determined using the kappa index. A kappa index ranges from 0 to 1 and the higher the value the stronger the agreement. All calculations were estimated at a 95% confidence interval (95% CI).

RESULTS

The Tb 927.1.4620(TB927-Target) gene was selected to prepare the *T. brucei* peptide Tb04.29m 18.750 used to immunize rabbits. The gene was specific for *Trypanozoon* as shown in Table 1 by Tb gene PCR. This is a family of related genes that shares a highly conserved N terminal and a variable C terminal. The fact that we are dealing with a family of genes with a conserved terminal N and variable C terminal may offer some interesting potential downstream application. The amino acid translated sequences of the genes are shown in Figure 1 and compared to the target sequence (TB927-Target).

Four of these genes (Tb927.1.4620 and orthologues clones 2, 3, and 4) were cloned and expressed. Rabbits were immunized with *T. brucei* peptide Tb04.29m18.750 and orthologues clones 2, 3, and 4 antigens. The antibodies produced in the rabbits were purified by chro-matography for assay. The antibodies were assem-bled into LFT pilot kits and tested against laboratory derived *T. brucei* procyclics (Figure 2). Antisera raised against the peptide were employed in assembling the lateral flow test kits. Best results were obtained with antiserum against the peptide Tb04.29m18.750. The test kit based on the antiserum was then evaluated against midguts from both

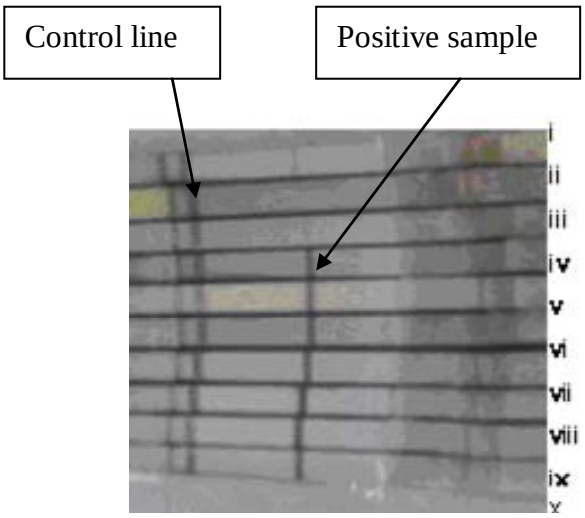

Figure 2. Homogenized tsetse midgut positive by microscopy is used as positive control sample , iv=positive control. Buffer reagents are used as negative samples seen in ii and iii=negative samples; all the rest are homogenized tsetse midguts which tested positive by microscopy in the field , I = weak positive sample and v-x=positive samples.

Table 1. Parasite Species/Subspecies results for the Tb927.1.4620 (TB927-Target) demonstrating the gene is specific to the *Trypanozoon.*

Isolate	Parasite species	Tb gene PCR
ILtat 1.1	*T.brucei*	+
ILtat 1.4	*T.brucei*	+
IL 1325	*T. b. gambiense*	+
IL2002	*T. b. rhodesiense*	+
IL1180	*T. congolense*	-
ILRAD 1895	*T. vivax*	-

Table 2. Evaluation of specificity of Lateral Flow Test using uninfected midguts from teneral and non-teneral tsetse flies.

Tsetse type	No. tested	No. Positive	No. Negative	Specificity %
Teneral tsetse midguts	45	0	45	100.0
Non-infected non-teneral tsetse midguts	25	0	25	100.0

The specificity of LFT using uninfected teneral and non-teneral tsetse flies was 100.0%.

from both experimentally infected tsetse and those from field collections.

Specificity of lateral flow test (LFT)

The LFT developed by Astel Diagnostics, Uganda, which is based on antibodies raised against the *T. brucei* peptide was evaluated for its analytical sensitivity, specificity, positive predictive value and negative predictive values in order to assess its utility for surveillance of trypanosome infections in tsetse flies. The evaluation of analytical specificity of lateral flow test using uninfected midguts from teneral and non-teneral tsetse flies was carried out and the results are summarized in Table 2.

Sensitivity of Lateral Flow Test (LFT)

The evaluation of analytical sensitivity of LFT using experimentally infected tsetse flies was carried out and

the results are summarized in Table 3. From these results, the sensitivity of LFT is 97.3%; specificity 74.0% and the test was statistically significant at P < 0.0001.

Performance of lateral flow test in detecting trypanosomes in wild tsetse flies from 6 countries

The diagnostic sensitivity, specificity, positive predictive and negative predictive values of lateral flow test were evaluated using wild tsetse midguts from 6 different countries (Table 4) and the test was statistically significant at P < 0.0001.

Performance of lateral flow test using a combined data from the 6 countries against microscopy

The diagnostic test agreement or concordance of the lateral flow test and microscopy was 91.0% and kappa value of 80.5% at the confidence interval 0.72 to 0.80 which is a high level of test agreement. Furthermore, the relative diagnostic sensitivity and specificity of lateral flow test were 83.0 and 96.0%, respectively. The positive and negative predictive values were at 92.7 and 90.0%, respectively which were high (Table 5). The lateral flow test is therefore recommended for surveillance of trypanosomes in tsetse flies in order to: 1) Indicate areas with tsetse infected with trypanosomes; 2) Indicate tsetse infected with potentially human pathogenic trypanosomes; 3) Guide in prioritizing control strategies for HAT. The lateral flow test is a rapid serological test that detects *T. brucei* trypanosomes in tsetse flies which transmit trypanosomes to humans through its bite. Its ease of applicability, use of crude extracts of tsetse midguts with no specialized equipment requirement will make it popular for use in typically rural poor settings. The proof of the principle of lateral flow test has shown that it has a great potential for monitoring current trypanosome infections in tsetse flies, thus indicating areas of high tsetse infection rates and risk of outbreak and spread of HAT.

DISCUSSION

Serological tests based on antigen-detection enzyme-linked immunosorbent assay (ELISA) have been, to a limited extent, applied for detecting trypanosomes in tsetse flies. Based on trypanosome-specific monoclonal antibodies developed for revealing trypanosomes in mammalian hosts (Nantulya et al., 1987), Bosompem (1993) developed a monoclonal antibody-based dot-ELISA for detecting trypanosomes in tsetse flies. This method was found to be more useful in differentiating culture-derived insect stages of major pathogenic trypanosomes (Bosompem et al., 1996). However, large scale application of these antigen-detection tests has been limited because they required that species specific antibodies be developed and tested for cross-reactivity against all trypanosome species.

This work, therefore, provides an option for a vast terrain infected with tsetse for surveillance targeting the vector with limited resources. This project was conceived to come up with a tool to facilitate prioritization control areas with high tsetse infection rates. The linkage in research with private partnership for development, production and dissemination of developed technologies to the rural poor at low costs was coined at the beginning of the project development. Astel diagnostics, Uganda, a private sector was engaged to work in collaboration with other partners to design a simple LFT for detecting trypanosomes in tsetse flies. The LFT is a rapid test that detects *T. brucei* trypanosomes in tsetse flies. The diagnostic test agreement or concordance of the lateral flow test and microscopy was 91.0% which is a high level of test agreement.

Furthermore, the diagnostic sensitivity and specificity of LFT were 83.0 and 96.0%, respectively. Both the positive predictive value at 92.7% and the negative predictive value at 90.0% were high. The proof of the principle of LFT has shown that it has a great potential for monitoring current trypanosome infections in tsetse flies. It is a cheap, simple and rapid test which gives results within 20 min, read by naked eyes and can be performed by personnel with minimal training. The use of the LFT for control activities will be guided by the prevailing circumstances. The subspecies of *T. brucei* in positive samples in the test can be confirmed using other available molecular tests (TgsGP-PCR and SRA-PCR) since LFT does not discriminate between *T.b. brucei, T.b, rhodesiesne and T.b. gambiense* all of which belong to the subspecies *T. brucei*.

In Uganda, prevention of the merging of rhodesiense and gambiense foci requires intensive integrated interventions. Keeping the two foci separate in Uganda is the most logical approach to control and elimination of African trypanosomiasis. There is need to keep the two foci apart while integrated control strategies applied in each focus. It is equally important to note that elimination of *T.b. rhodesiense* is more complicated because of its zoonotic transmission which requires involvement of tsetse vector control. Thus the LFT has application in checking infections in the buffer zone that should be demarcated between the 2 endemic foci.

In Tanzania, strategic control of tsetse and African Trypanosomiasis in these areas will lead to significant contribution to improving human health, livestock sector and boost tourism through tsetse control in areas with tsetse pathogenic trypanosomes. *T.b. rhodesiense* sleeping sickness in Tanzania has to date been sporadic and of low endemicity in Tanzania. However, in the previous years, there were reports of epidemics that suggest that the situation of the disease has changed probably due

Table 3. Comparison of microscopy and LFT using experimentally infected tsetse.

Lateral flow test	Microscopy results on samples of *T. brucei* infected tsetse guts			Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Level of significance
	Positive	Negative	Total					
Positive	72	14	86					
Negative	2	40	42	97.3	74.0	83.7	95.2	P<0.0001
Total	74	54	128					

PPV= Positive predictive value, NPV= Negative predictive value.

Table 4. Performance of lateral flow test in detecting infections in wild tsetse flies

Country	Lateral flow test results	Microscopy results on samples of midguts			Sensitivity	Specificity	PPV	NPV	Level sign
		Positive	Negative	Total					
Tanzania	Positive	21	1	22	75.0	97.5	95.5	84.8	P<0.0001
	Negative	7	39	46					
	Total	28	40	68					
DRC	Positive	20	0	20	80.0	100.0	100.0	88.9	P<0.0001
	Negative	5	40	45					
	Total	25	40	65					
Malawi	Positive	36	4	40	90.0	90.0	90.0	90.0	P<0.0001
	Negative	4	36	40					
	Total	40	40	80					
Sudan	Positive	31	0	31	77.5	100.0	100.0	81.6	P<0.0001
	Negative	9	40	49					
	Total	40	40	80					
South Sudan	Positive	34	6	40	85.0	85.0	85.0	85.0	P<0.0001
	Negative	6	34	40					
	Total	40	40	80					
Uganda	Positive	24	2	26	88.9	98.3	92.3	97.5	P<0.0001
	Negative	3	118	121					
	Total	27	120	147					

Sens=Sensitivity, Spec= Specificity, PPV= Positive predictive value, NPV= Negative predictive value, Level sign. = Level of significance.

Table 5. Performance of Lateral Flow test using combined data from all countries against microscopy as a gold test

	Lateral flow test results	Microscopy test results		Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Test agreement (%)
		Positive	Negative						
All countries	Positive	166.0	13.0	179.0					
	Negative	34.0	307.0	341.0	83.0	95.9	92.7	90.0	90.9
	Total	200.0	320.0	520.0					

Positive predictive value=PPV, Negative predictive value= NPV

changed probably due to ecological as well as socio-demographic changes favouring development of new active foci hence a need for instituting sustainable, user and environmental friendly techniques against the vectors of African Trypanosomiasis. Thus applying xenomonitoring tools such as LFT will be useful to monitor and predict the eminent outbreaks of HAT for timely intervention of control activities against tsetse to prevent and or minimize the outbreaks of sleeping sickness in the area would be a feasible option. The documented HAT cases of tourists together with local park residents infected with *T.b. rhodesiense* demonstrate the need to halt transmission by identifying areas with tsetse flies having pathogenic trypanosomes to humans and animals for targeted control.

In Malawi, tourists together with local population around the National Parks and the Game Reserves have been infected with *T.b. rhodesiense* and this has a negative effect on tourism industry as well as on the health of the population. There is, therefore, need to reduce the incidence of infection and prioritize control strategies in and around National Parks and Game Reserves by regular use of xenomonitoring tools to detect trypanosomes in tsetse flies**.** Besides, there is emerging evidence that tsetse flies are highly infected with *T. brucei* from this study where in some cases up to 85% of tsetse flies caught in Liwonde National Park were habouring *T. brucei* infections yet no recorded case of HAT has been reported in and around Liwonde National Park to our knowledge. This could be due to lack of active surveillance for HAT in human population in the areas.

Recently, foreign visitors to Kasungu National Park have reported to have been infected with HAT. Also the HAT cases caused by *T.b. rhodesiense* in Malawi are subacute as most people infected present themselves passively to district hospitals mostly in second stage of the disease (MacLean et al., 2010). The case fatality rates are very high due to this fact (Chisi et al., 2011). Furthermore, there are very few studies in Malawi that have concentrated on the role that tsetse flies play on the cycle of HAT transmission. The Congo River of Democratic Republic of Congo and Congo Brazzaville trans border is an active Gambiense HAT transmission area with Bandundu and Ngabé HAT foci. This socioeconomically active zone would benefit on surveillance strategy using xenomonitoring tools such as LFT. In addition, at Congo Brazzaville one could monitor spreading of trypanosomiasis from Congo River foci to neighboring trypanosomiasis free areas using LFT.

Most detection techniques for trypanosomes require technical knowhow in their use, hence restricted to developed labs, requires refrigeration which is non-reliable in rural areas where infected tsetse organs are collected, and some essential reagents are not readily and easily available to execute different trypanosome detection techniques. In this paper we have highlighted the need to come up with simple, user friendly, ultra-sensitive technique which can be used to detect trypanosomes especially the human infective ones as an early warning tool to predict the outbreak of tsetse borne diseases in human beings. Future work may include development of a LFT that discriminate between *T. brucei* subspecies and or *T. congolense* and *T. vivax*.

ACKNOWLEDGEMENTS

We are greatly indebted to Global Heath of the Bill and Melinda Gates Foundation for the financial assistance and to all its staff members who entirely assisted and encouraged us during the implementation of this project for five years. Secondly, we would like to thank the participating institutions: Tsetse and Trypanosomiasis Research Institute (TTRI), Tanzania, Tropical Medicine Research Institute (TMRI) Sudan, Ministry of Animal Resources and Fisheries South Sudan, College of Medicine, University of Malawi, The National Program of Control of Human African Trypanosomiasis (NPHAT), Congo

Kinshasa, Astel Diagnostics in Uganda especially their Technical and Administrative personnel in each of the mentioned institutions. Thirdly, we thank the Makerere University Administration and the Department of Biochemistry and Sports Science in particular for hosting the project so well for the last five years. Lastly, but not least, the Uganda National Council of Science and Technology, (UNCST), the Ministry of Health, the Ministry of Agriculture, Animal Industry and Fisheries for the ethical clearance and permission to carry out the research.

Conflict of interests

The authors declare that there is no conflict of interests

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